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TITLE: Is Peripheral Benzodiazepine Receptor (PBR) Gene Expression Involved in Breast Cancer Suppression by Dietary Soybean Protein

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**The objective was to produce breast cancer model in female rats, fed casein containing diet, by gavage administration of DMBA, and to investigate whether the tumor development is controlled by replacement of casein with soybean protein. Two separate experiments were done. For each experiment, Twenty 21-day old female Sprague Dawley rats were used. They were divided into 4 groups. Animals from groups 1 and 2 received standard AIN-76A diet containing 20% casein and those of groups 3 and 4 received same diet containing 20% soybean protein. Animals of groups 2 and 4 received DMBA in sesame oil by gavage (15 mg per animal). Control animals (groups 1 and 3) received the vehicle. Animals were weighed and palpated twice weekly. At the end of the study (postinjection time of 122 days), the animals were killed by carbon dioxide asphyxiation. All tumors were detected by palpation and at autopsy biopsy specimens were taken for histological analysis. Breast tissues were removed, quickly frozen in liquid nitrogen and stored at –80°C for biochemical analysis. Even though multiple tumors were observed in some animals of both groups, the number of tumors per rat was less in soyprotein group than casein group at any time point after DMBA exposure. Incidence of tumors was less in soybean protein group than that in casein group. There was no tumor in any animal which did not receive the carcinogen. Casein group had 20% grade I, 60% grade II and 20% grade III mammary gland adenocarcinoma. However, the soybean group had 100% grade I adenocarcinoma and no aggressive grade II or III. These findings suggest that the soybean protein may protect against the development of a more aggressive mammary gland adenocarcinoma. Furthermore, there was a delay in the development of adenocarcinoma in the soybean group challenged with DMBA in comparison to the animals fed casein and challenged with DMBA.**

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## INTRODUCTION

**The beneficial effects of dietary soybean protein in human health, particularly in breast cancer prevention, have been recently emphasized. However, to our knowledge, no information is available concerning the effects of dietary consumption of soybean protein on the expression of some genes, which may play a vital role in the prevention of breast cancer. It has recently been shown that ligand binding and mRNA expression of peripheral benzodiazepine receptor (PBR) is dramatically increased in the highly aggressive breast cancer cell lines and aggressive metastatic human breast tumor biopsies compared with nonaggressive cell lines and normal breast tissues. PBRs in aggressive breast cancer cell lines and tissue biopsies are mostly localized in and around the nucleus, which is in contrast to the largely cytoplasmic localization in nonaggressive cell lines and normal breast tissues. Furthermore, in aggressive cell lines, PBR drug ligands are found to increase the uptake of cholesterol by the nuclei and simultaneous incorporation of bromodeoxyuridine into the cells, suggesting the role of PBRs-mediated nuclear cholesterol uptake in cell proliferation. Numerous studies also implicate a role of nuclear cholesterol in the mechanisms underlying cell proliferation and cancer progression. It is not known whether the beneficial effect of dietary soybean protein on breast cancer suppression is mediated by its inhibitory effect on PBR expression, nuclear localization, and PBR-mediated cholesterol transport into the nucleus and cell proliferation. The objective of this project is to test the hypothesis that increased ligand binding, increased gene expression and possible mutation(s), and nuclear localization of PBRs, and PBRs-mediated cholesterol transport into the nucleus of breast epithelial cells are involved in cancer proliferation, and this aggressive phenotype expression can be prevented by dietary consumption of soybean protein.**

## WORK DONE DURING THE FIRST YEAR (May 1, 2003 – April 30, 2004)

### APPROVED STATEMENT OF WORK

**Task 1.** To develop a breast cancer model by administration of DMBA to female rats fed a diet containing casein as the source of protein, and to inhibit the tumor development with soybean as the dietary protein (Months 1-18).

a. Feed weanling animals standard diets containing either casein or soybean protein and give DMBA by gavage and maintain the animals for 80 days.
b. Confirm breast cancer development in animals fed casein and suppression of breast cancer in animals fed soybean protein after 80 days of feeding.
c. Collect breast tissue for biochemical studies.
d. Develop and standardize methodologies for biochemical assays.

**Task 2.** To determine the role of PBRs in breast cancer suppression by dietary soybean protein (Months 18-36)

a. Maintain primary cultures of breast epithelial tissues.
b. Assay of PBR ligand binding in breast epithelial cells (Specific Aim 1).
c. Localize PBRs in cell nuclei by fluorescent microscopy (Specific Aim 2).
d. Measure nuclear uptake of cholesterol in breast epithelial cells (Specific Aim 3).
e. Measure bromodeoxyuridine uptake by breast epithelial cells (Specific Aim 4).
f. Measure ornithine decarboxylase activity of breast epithelial cells (Specific Aim 4).
g. Quantitate the expression c-fos in breast epithelial cells (Specific Aim 4).
h. Quantitate the expression of mRNA for PBRs in breast epithelial cells (Specific Aim 5).
i. Sequencing of the full-length cDNA for PBRs from breast epithelial cells (Specific Aim 6)

During this period we focused our studies primarily on Task 1 as follows:

### Development of Breast Tumor in Rats

#### Design of Animal Study

The first objective of this study was to produce breast cancer model in female rats, maintained on casein containing diet, by gavage administration of DMBA, and to investigate whether the tumor development can be counteracted by replacement of casein with soybean as dietary source of protein. So far we have carried out two separate experiments. For each experiment, 20 female Sprague Dawley rats were obtained at 21 days of age. They were divided into 4 groups. Animals from groups 1 and 2 received standard AIN-76A diet containing 20% casein and those of groups 3 and 4 received

same diet containing 20% soybean protein instead of 20% casein. Diets were prepared by Harlan Teklad, WI and the composition of diets is given in Table 1. The diets were designed to be similar in nutrient content.

**TABLE 1. Composition of Diets**

| Ingredients | Casein (g/kg) | Soybean Protein (g/kg) |
|---|---|---|
| Casein | 200.0 | - |
| Soy Assay Protein | - | 200.0 |
| L-Cystine | 3.68 | 1.88 |
| DL-Methionine | - | 2.32 |
| Sucrose | 482.7273 | 493.586 |
| Corn Starch | 150.0 | 150.0 |
| Corn Oil | 50.0 | 44.2 |
| Cellulose | 50.0 | 50.0 |
| Mineral Mix, AIN-76 (170915) | 35.0 | 35.0 |
| Calcium Carbonate | 9.68 | 11.004 |
| Cupric Carbonate | 0.0057 | - |
| Ferric Citrate | 0.156 | - |
| Sodium Bicarbonate | 6.124 | 6.124 |
| Vitamin Mix, AIN-76A (40077) | 10.0 | 10.0 |
| Choline Bitartrate | 2.617 | 2.0 |
| Ethoxyquin (antioxidant) | 0.01 | 0.01 |

Animals received food and water ad libitum. Animals of groups 2 and 4 received DMBA dissolved in sesame oil by gavage (15 mg per animal). Control animals (groups 1 and 3) received the vehicle only by gavage. Animals were weighed and also palpated twice weekly to detect breast tumors beginning four weeks after the administration of carcinogen. At the end of the study (postinjection time of 122 days), the animals were killed by carbon dioxide asphyxiation. All tumors were detected by palpation and at autopsy biopsy specimens were taken for histological analysis. Breast tissues were removed, quickly frozen in liquid nitrogen and stored at –80°C for direct biochemical analysis.

**Results:**

**Body Weights.** DMBA injection had no significant effects on the growth of animals. Furthermore, the growth pattern was similar in animals fed either casein or soybean protein (**Fig. 1**).

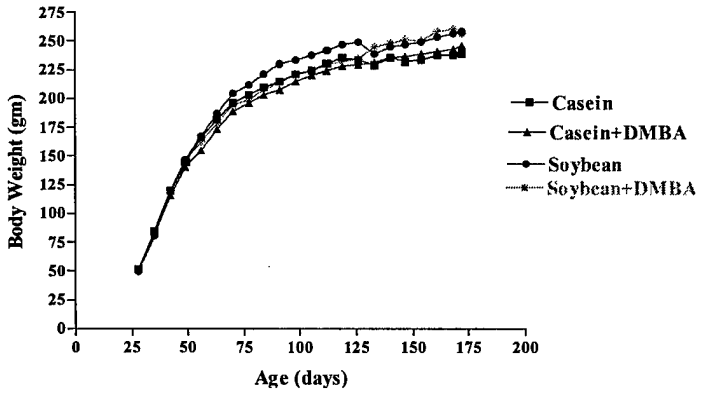


**Fig. 1. Effects of DMBA injection on body weight gain of rats fed either casein or soybean protein.**

## Tumor Development

**Time Course for Tumor Formation.** The time course of palpatable breast tumor appearance is shown in **Fig. 2**. Even though multiple tumors were observed in some animals of both groups, the number of tumors per rat was less in soyprotein group than casein group at each postinjection time.

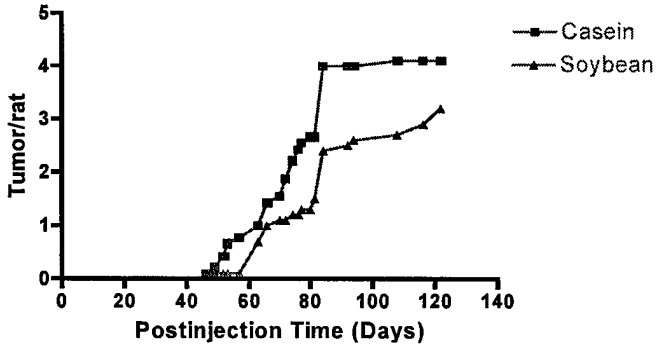


**Fig.2. Time Course of Palpatable Breast Tumor**

**Breast Tumor Incidence.** Breast tumor incidence (percentage of rats with tumors) of female rats is shown in **Fig. 3**. Incidence of tumors was less in soybean protein group than that in casein group.

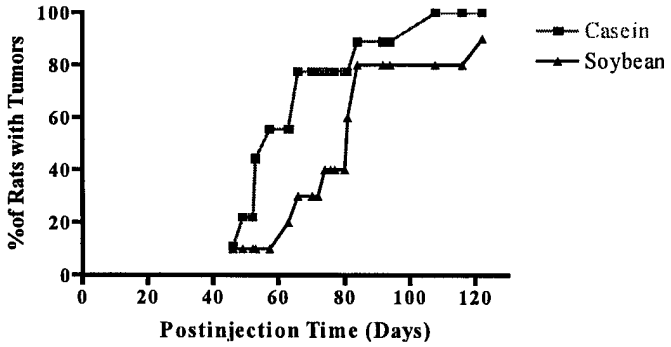


**Fig. 3. Breast Tumor Incidence**

## Tumor Characteristics

There was no tumor in any animal which did not receive the carcinogen regardless of whether they were fed casein or soy protein. Even though there was a difference in the time course of tumor development between the casein group and the soyprotein group, the tumors were visibly apparent externally for both groups (**Fig. 4**).

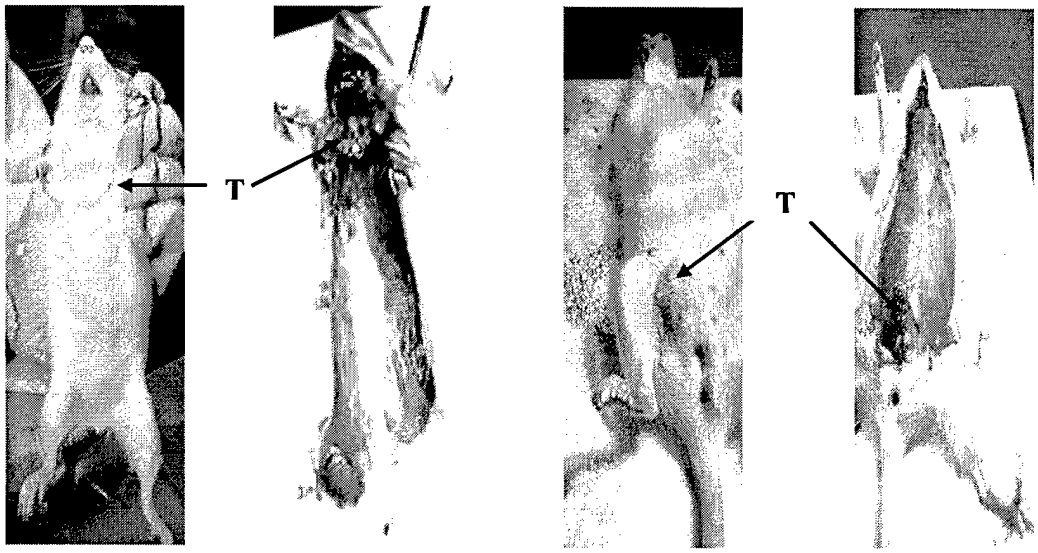


**Fig. 4. Development of Breast Tumors (T) in Animals Fed Casein and Soy Protein**

Some tumors in both groups had darker area, possibly implicating cessation of angiogenesis (CAn) in that area (**Fig. 5**). However, the degree of discoloration was higher in animals fed soyprotein than that in animals fed casein. **It is important that we investigate whether soyprotein retards the progression of angiogenesis**. Furthermore,

while the size of the largest tumor was not remarkably different between soyprotein group (3.5mm x 3.3mm x 1.2mm) and the casein group (3.8mm x 2.78mm x 1.6mm), the weight of the largest tumor was 44.5% lower in soybean group (7.52 g) than that in the casein group (13.54 g). It will be of interest to find out whether the tumors will shrink if the animals are fed soyprotein diet for longer period of time.

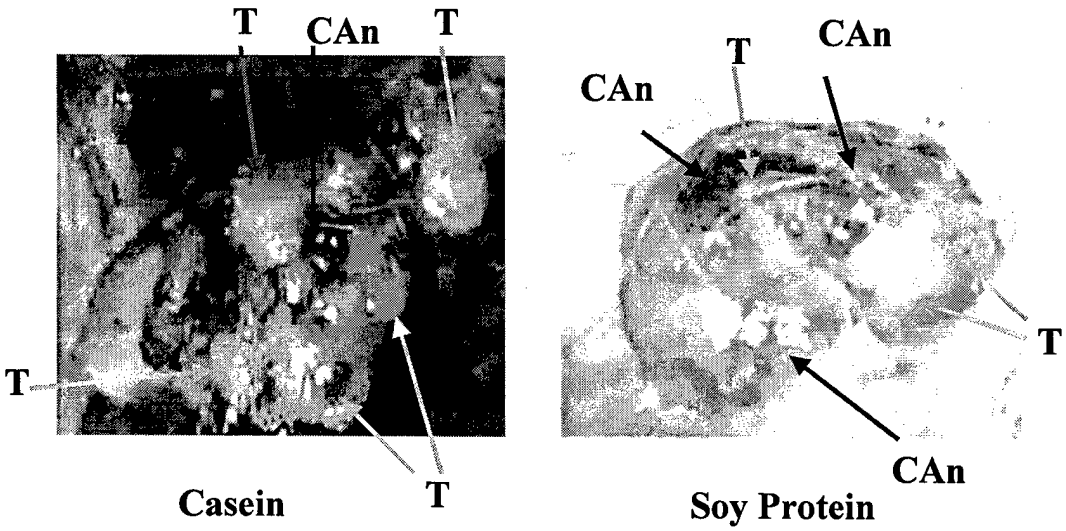


**Fig. 5. Possible Cessation of Angiogenesis (CAn) in Breast Tumors (T) in Animals**

Control animals had visible blood vessels (**Fig. 6**). However, in soybean group, there was a dilation of blood vessels which may stimulate Angiogenesis.

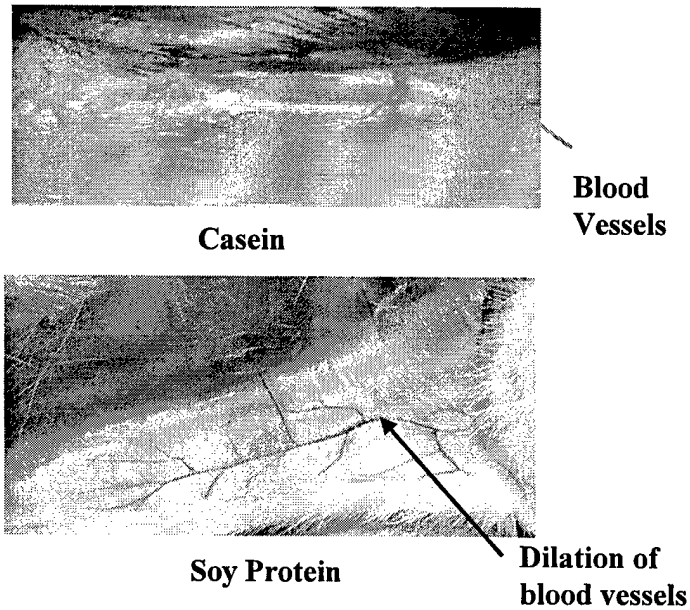


**Fig. 6. Dilation of Blood Vessels in Animals Fed Soy Protein**

## Pathology of Breast Tumors

So far thirty-one specimens from animals treated with DMBA were examined microscopically. Seventeen of these specimens were from the soybean group and 14 specimens were from the casein group. In the soybean group, two specimens did not demonstrate a mammary gland neoplasm and 15 specimens demonstrated a grade I mammary gland adenocarcinoma. None of the specimens in this group demonstrated either grade II or grade III mammary gland adenocarcinoma. In the casein group, 4 specimens did not demonstrate a mammary gland neoplasm, 2 had a grade I, 6 had a grade II and 2 had a grade III mammary gland adenocarcinoma. **Thus, while 100% of the mammary gland adenocarcinoma found in the soybean group was of the non-aggressive type (grade I). However, in the casein group, there was a higher percentage of aggressive tumors (20% grade I, 60% grade II, 20% Grade III).** Representative photographs of light microscopy from grade I tumor from the soybean group and grade II and grade III tumor from the casein group are shown in Fig. 7, 8 and 9, respectively. Representative photograph of light microscopy from breast tissue of control animals regardless of whether fed casein or soybean protein is shown in Figure 10.

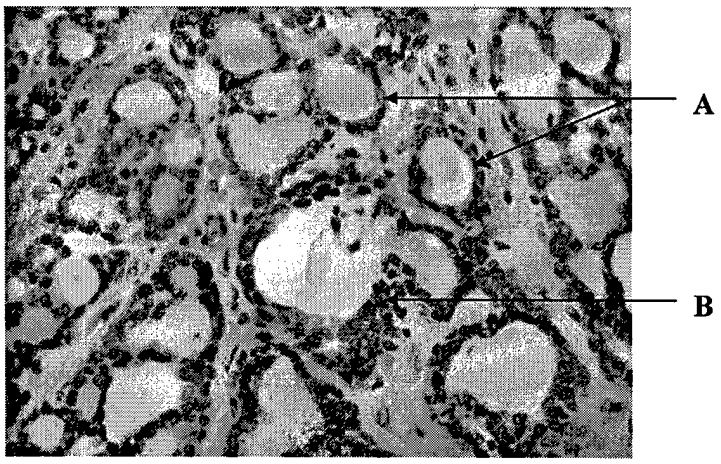


**Fig. 7.** Histological sections from breast of rats fed soyprotein and treated with DMBA. Development of grade I adenocarcinoma exhibiting well defined ducts (A), small regular uniform nuclei (B), and less than 9 mitoses per 10 high power fields (40X)

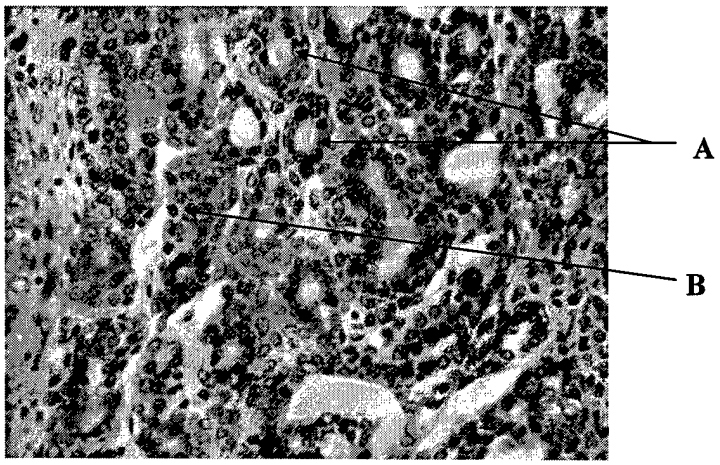


**Fig. 8.** Histological sections from breast of rats fed casein and treated with DMBA. Development of grade II adenocarcinoma exhibiting equal number of ducts (A) and solid areas (B), 40X

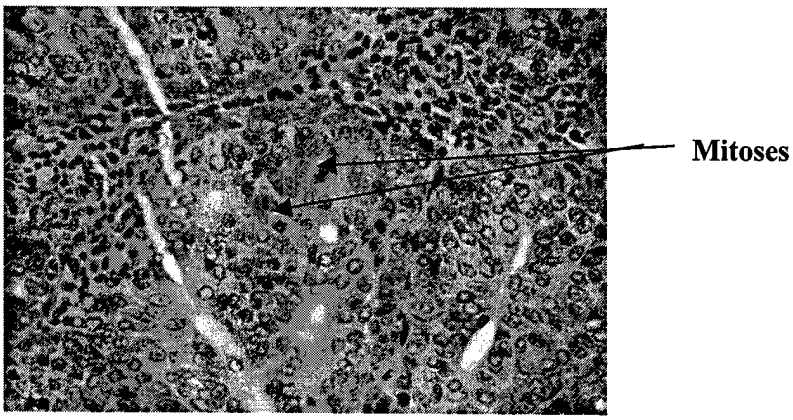


**Fig. 9.** Histological sections from breast of rats fed casein and treated with DMBA. Development of Grade III adenocarcinoma exhibiting solid sheets of malignant cells with crowded, pleomorphic, hyperchromatic nuclei and prominent eosinophilic nucleoli and more than 20 mitoses per 10 high power fields (40X)

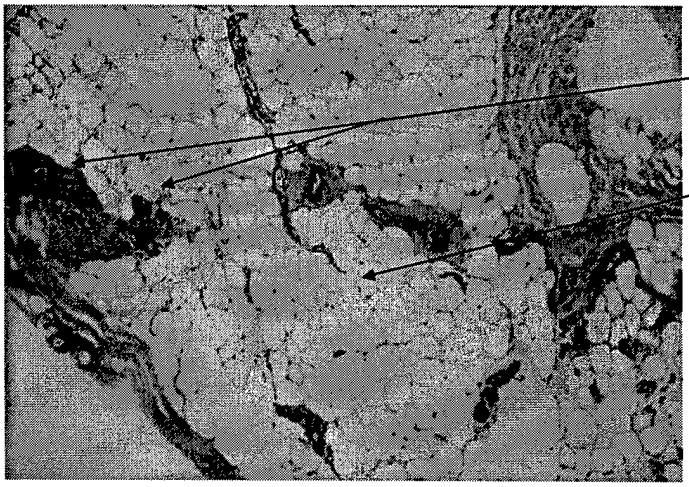


**Fig. 10**. Histological sections from normal breast of rats fed casein showing duct and lobules (A) and adipose tissue (B), 10X.

**Discussion**:

The histologic grading of mammary gland carcinoma is a method used by pathologist to assess and predict the aggressiveness and clinical behavior of human mammary gland adenocarcinoma. This system is based on the tubule formation within the neoplasm, nuclear pleomorphism, and mitotic count per high power field (3). Using these parameters mammary gland adenocarcinoma are graded I, II, and III. Grade I is the least aggressive and has the best prognosis and grade III is the most aggressive and has the worst prognosis. The specimens from the soybean group and the casein group of animals were graded. Casein group had 20% grade I, 60% grade II and 20% grade III mammary gland adenocarcinoma. However, the soybean group had 100% grade I adenocarcinoma and no aggressive grade II or III.

These findings suggest that the soybean protein may protect against the development of a more aggressive mammary gland adenocarcinoma. Furthermore, there was a delay in the development of adenocarcinoma in the soybean group challenged with DMBA in comparison to the animals fed casein and challenged with DMBA.

**Isolation of Rat Mammary Epithelial Cells**

After sacrificing, 6 pairs of mammary gland (bilateral cervical, cranial thoracic, caudal thoracic, abdominal, cranial linguinal and caudal linguinal) from each animal were excised and minced. The minced glands were digested for about 15 h with a sterile solution containing DMEM/F12 medium with FBS, gentamicin, class 3 collagenase, and grade II dispase (4). The upper lipid layer was aspirated away and the remaining suspension was centrifuged at 500 xg for 10 min at 4°C. The pellet was resuspended in

adherence medium containing 5% FBS, DMEM-F12 and gentamicin, and the cell suspension was filtered through a 530μm nitex filter. The filtrate was passed through a 60μm filter. The filtrate was incubated in a 37°C humidified $CO_2$ incubator for 4h. The non-adherent cell suspension was recovered and centrifuged. The resulting pellet was suspended in DMEM-F12 medium containing insulin, prolactin, EGF, progesterone, hydrocortisone, apo-transferrin, ascorbic acid, fatty-acid-free BSA and gentamicin for culturing the cells. Light microscopic photographs of normal rat mammary epithelial cells and mammary epithelial organoids (MEO) are shown in **Figure 11 and 12,** respectively.

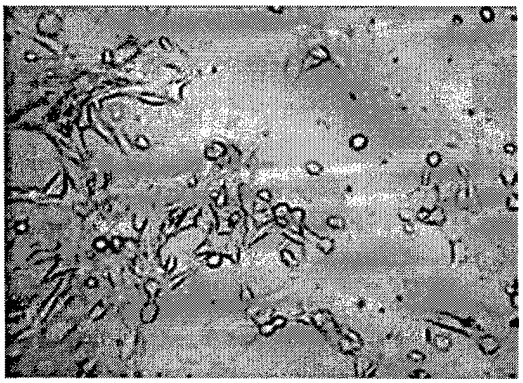

**Fig. 11**

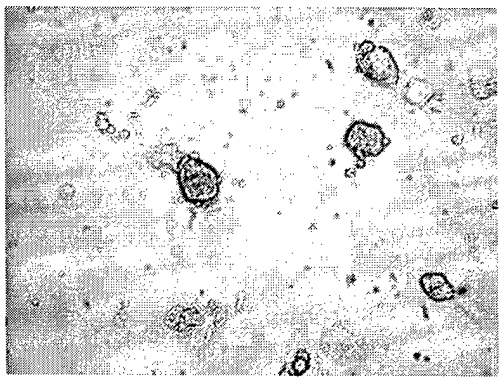

**Fig. 12**

**Key Research Accomplishment:**

1. Development of breast cancer model in rats by dietary feeding of casein as source of protein and administration of DMBA by gavage. Majority of cancers was of aggressive type (60% grade II and 20% grade III). Only 20% was of the grade I (non-aggressive) type.
2. Dietary consumption of soybean protein has a beneficial effect. It delays the onset of cancers and also produces a less aggressive cancer (100% non-aggressive). Therefore, prognosis will be better if soybean protein is consumed in lieu of casein as dietary source of protein.
3. Even though both casein and soybean fed animals had breast cancer when gavaged with DMBA, soybean protein consumption possibly retards the progression of Angiogenesis.
4. We have standardized the methods of isolation and culturing of rat mammary epithelial cells.
5. We have standardized all techniques in relation to the binding assay for peripheral Benzodiazepine receptors.
6. We have standardized the methods of nuclear cholesterol transport assay.

## Reportable Outcomes:

### Manuscript:

1. Differential Effect of Cadmium on Cholinephosphotransferase Activity in Normal and Cancerous Hhuman Mammary Epithelial Cell Lines, SinhaRoy, S., Mukherjee, S., Mukhopadhyay, S., Das, S. K., Mol Cancer Ther. 2004; 3(2): 199-204.
2. Expression of Mn-Superoxide Dismutase Gene in Non-Tumorigenic and Tumorigenic Human Mammary Epithelial Cells, In Press, J. Biomedicine Biotechnology.

### Abstract:

1. Correlation between Expression of the Peripheral Benzodiazepine Receptors and Cancer Cell Proliferation, Akech, J. and Das, S.K., FASEB J. 2004, 18(8): C109.

## Conclusions:

Current results support the idea that soybean protein has a beneficial effect in controlling the aggressiveness in breast cancer progression. While, 100% of the breast tumor induced in rats fed soybean protein was of grade I (non-aggressive) type, casein consumption produced more aggressive tumors (20% grade I, 60% grade II and 20% grade III).

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